Review

Host subversion by formation of intracellular bacterial communities in the urinary tract

Gregory G. Anderson, Steven M. Martin, Scott J. Hultgren*

Department of Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Avenue, Campus Box 8230, St. Louis, MO 63110, USA

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Abstract

Urinary tract infections pose a serious health threat with respect to antibiotic resistance and high recurrence rates. While the host robustly responds to bacterial infiltration into the bladder, uropathogenic Escherichia coli can survive the onslaught to persist for months after initially infecting. To accomplish this feat, uropathogenic E. coli forms intracellular bacterial communities, with many biofilm-like properties, within the bladder epithelium. These communities may allow bacteria to subvert host defenses and form a persistent reservoir in the bladder.
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1. Introduction

Urinary tract infections (UTIs) affect a large proportion of the world population and are one of the most common infectious diseases [1]. It is estimated that a majority of women will have at least one UTI some time in their life, and many women experience multiple, recurrent infections. In addition to women, UTIs can cause serious morbidity in the elderly and in young children [2]. The sheer numbers of UTI have created a huge economic burden for evaluation and treatment, with medical expenditures nearing $2 billion in the United States alone [1]. Clinically, UTIs are defined as “complicated” or “uncomplicated” [1]. Complicated UTI refers to infections in patients with obstructed or abnormal urinary tracts, or with medical instrumentation, such as a urinary catheter. Uncomplicated UTI describes infection in patients with normal urinary tracts and without instrumentation. Cystitis (bladder infection) and pyelonephritis (kidney infection) are the disease states that are most often encountered in the clinic, but there are a wide variety of other clinical syndromes, including bacteriuria, prostatitis, urethritis, and asymptomatic bacteriuria [3]. Symptoms associated with these conditions include painful, frequent, and urgent urination, suprapubic pain, lower back pain, nausea, vomiting, fever, pyuria, and hematuria [3]. In some cases, UTIs can lead to sepsis and even death [2]. Perhaps most frustrating, however, is the high rate of recurrence of symptoms, even in patients with uncomplicated UTI, and despite antibiotic therapies. Nearly 26% of women with an acute UTI will have at least one recurrence within 6 months of an initial UTI, and many have multiple recurrences [1]. The most common infecting organism among all clinical disease states of UTI is uropathogenic Escherichia coli (UPEC), which is found in 70–95% of all uncomplicated cases [3]. UPEC colonizes and persists in the urinary tract by taking advantage of urinary tract biology.

2. Bladder biology

Infecting organisms are thought to ascend the urinary tract, gaining entry at the urethra, ascending into the bladder, and then possibly through the ureters into the kidneys [4]. The bladder is unique as a major organ, whose sole purpose is to collect and expel urine. As it does this, the bladder encounters the problem of preserving its epithelial integrity during constant expansion and contraction. The epithelium must resist these mechanical forces encountered during normal bladder function and maintain a permeability barrier to pre-
vent leakage or diffusion of solutes and toxins concentrated in the urine into epithelial tissues. To solve this problem, the bladder constructs a transitional epithelium with several fascinating characteristics [5]. Separated from the muscle wall by a thin lamina propria, the epithelium is divided into three layers: basal, intermediate, and superficial. The intermediate cells are formed by upward extension of the basal cells. Fusion of intermediate cells gives rise to large superficial cells, which can be 100 μm in diameter, or greater, leading to the designation “umbrella cells” [6]. These umbrella cells are terminally differentiated, and provide the main functional basis for the bladder permeability barrier. On their luminal surface, the superficial cell’s role is expressed integral membrane proteins called uroplakins (UP). There are four UP molecules, UPIa, UPIb, UPII, and UPIII that associate into hexameric rings. These hexameric rings are organized into crystalline plaques, which cover most of the luminal surface of the superficial cell. Because the bulk of the UP’s structure is found in the outer leaflet, the luminal membrane has been termed the “asymmetric unit membrane” [6]. The UP plaques on the surface of the bladder epithelium add strength to the epithelial membrane, preserving physical membrane integrity as the bladder expands and contracts. In addition, fusiform vesicles containing additional UP-coated membrane reside just underneath the luminal surface, and fuse with the membrane upon bladder expansion to quickly provide increased bladder surface area [6]. The UP plaques also create a chemically impermeable layer, effectively blocking the diffusion of luminal contents into the epithelium [6]. Another major factor influencing the permeability barrier is the extremely low turnover rate of the bladder epithelium, which is estimated at only 6–12 months in humans [5]. Although many signaling pathways exist to initiate and carry out epithelial regeneration and renewal, these are normally repressed in the bladder epithelium [7]. This slow bladder turnover aids bladder function by keeping the vital UP-coated asymmetric unit membrane intact. Upon sloughing of a superficial cell, the repressed developmental pathways are activated in the intermediate cells, which then quickly fuse together and differentiate to replace the superficial cell [5,7]. In this manner, the bladder can preserve an intact superficial epithelium.

3. Early events in UPEC pathogenesis

UPEC expresses a number of adhesins, which allow it to attach to urinary tract tissues [8]. These adhesins are classified as either fimbrial or afimbrial, depending upon whether or not the adhesin is displayed as part of a rigid pilus (or fimbria). Fimbrial adhesins implicated in urinary tract pathogenesis include P pili, type 1 pili, and S pili. P pili confer tropism to the kidney, the designation “P” standing for pyelonephritis [8]. The adhesin PapG, at the tip of the pilus, recognizes and binds globoside found on the human kidney epithelium. The binding site on PapG is on the side of the molecule, and recognition of its receptor is thought to be aided by a long, flexible linker region on the pilus just below the adhesin subunit, which permits the adhesin to be rotated into a binding-competent orientation [9]. Type 1 pili are vital for initiation of bladder infection. Similar to P pili, type 1 pili incorporate an adhesin, FimH, at their distal tip. FimH recognizes mannose, and in the bladder FimH binds to mannosylated residues on the UP molecules [10]. S pili have been associated with both bladder and kidney infection, and these pili utilize the adhesin SfαS to bind to sialic acid residues [8]. UPEC also expresses a group of related afimbrial adhesins (AFA), which have been clustered with the Dr adhesin family for their recognition of decay-accelerating factor and for their similar genetic structure [8]. Decay-accelerating factor is found on numerous different epithelial sites, and Dr adhesins are known to bind at many locations throughout the urinary tract.

In general, these fimbrial and AFAs are assembled by the chaperone/usher pathway [11]. In the chaperone/usher scheme, a periplasmic chaperone binds and stabilizes nascent pilus subunits as they are translocated into the periplasm by the general secretory pathway. Each subunit has the structure of an incomplete immunoglobulin fold. The chaperone donates a β-strand to fit into a groove on the pilus subunit. This interaction completes the immunoglobulin fold, assists folding of the subunit, and shields subunit inter-active surfaces. The chaperone/subunit complex traverses the periplasm to the outer membrane, where the chaperone is displaced and the subunit–subunit interactions occur in a defined manner so that the hydrophobic core of each subunit is completed by the N-terminal strand of the next distal subunit. The pilus rod is then built from the bottom up, traversing the outer membrane via the usher protein, and numerous subunits are added to create a rigid structure extending from the bacterial surface.

As mentioned above, UPEC uses type 1 pili to colonize the host bladder epithelium (Fig. 1A). Expression of type 1 pili is regulated by a form of phase variation whereby two recombinases, FimB and FimE, influence the orientation of an invertible switch in the promoter region of the pilus operon [12]. In human and animal studies of UTI caused by UPEC, bacteria associated with bladder cells generally have their type 1 pilus promoter oriented in the phase ON position, competent for pilus expression [13,14]. However, a large percentage of the bacteria in the urine are in the phase OFF orientation, which abrogates pilus expression. In studies using a mouse model of cystitis, bacterial mutants locked in the phase ON orientation are recovered from the urine and bladder in greater numbers compared to wild-type organisms [12]. Conversely, bacterial mutants locked in the phase OFF orientation are severely defective for bladder colonization. These studies underscore the importance of type 1 pili for maintenance of UTI, and it seems likely that the ability to switch between ON and OFF orientations may influence transmission and spread of the organism across the urinary tract and between hosts.
The actual epithelial recognition occurs via the FimH adhesin at the distal tip of type 1 pili. The mannose binding site on FimH, which exists as a deep acidic pocket at the tip of the molecule, is highly conserved across urinary tract isolates of *E. coli* [10]. FimH binds mannosylated residues on the UP molecules covering the bladder superficial epithelial cells. While numerous wild-type UPEC can be seen randomly attached to the epithelium immediately after infection, an isogenic fimH-mutant cannot colonize mouse bladders [15]. High-resolution freeze-fracture electron microscopy has shown that the tips of these pili, including the adhesin, are buried in the central cavity of the UP hexameric rings [15]. In this manner, UPEC exploits host biology to target the bladder and tether itself to the epithelium (Fig. 1A).

Soon after attachment to the epithelium, UPEC is quickly internalized into the bladder superficial cells [16] (Fig. 1A, B). Studies on the mechanisms of invasion, carried out in immortalized human bladder epithelial cells in vitro, have established FimH as the key to UPEC invasion. Isogenic fimH-mutants do not invade, and invasion of wild-type bacteria can be inhibited by addition of mannose. Further, polystyrene latex beads coated with FimH are quickly internalized in a process identical to bacteria expressing type 1 pili. Upon FimH-mediated attachment, host activation of the Rho-family GTPases RhoA, Cdc42, and Rac1 leads to recruitment of focal adhesin kinase, phosphoinositide-3 kinase, α-actinin, and vinculin [16]. These host processes result in localized actin rearrangements and engulfment of the bound bacterium by zipperring of the membrane around the microorganism. Invasion into the bladder superficial epithelium allows UPEC to establish a new niche as part of an effort to conceal itself from a strong host innate immune response.

### 4. Host response to UPEC infiltration

Mammalian responses to invading pathogens are a set of complex processes designed to eliminate the microorganism from the host. The mechanisms involve both adaptive and innate immunity. Adaptive immunity, involving specific recognition of pathogens by T and B lymphocytes and production of high-affinity antibodies, develops over 7–10 days. The innate immune response occurs more rapidly than adaptive immunity and involves responses from a variety of cell types, including neutrophils, macrophages, eosinophils, natural killer (NK) cells, mast cells, and dendritic cells (DCs). The innate response aids in establishing adaptive immunity due to interactions of macrophages, DCs, and NK cells with T and B lymphocytes.

The ability of the host to quickly recognize a pathogen is mediated by a series of receptors termed pathogen pattern recognition receptors. These specialized receptors, which come from several receptor families, recognize molecular patterns that are conserved across many species of pathogens, such as lipopolysaccharide (LPS) and peptidoglycan. The major pathogen pattern recognition receptors for bacteria are the toll like receptor (TLR) family. Until recently, there have been only 10 TLR genes described [17]. The best-studied TLR is the LPS receptor, TLR4. LPS recognition by TLR4 requires several additional proteins, including
CD14, MD-2, and LPS binding protein [17]. TLR4 signaling induces cytokine and chemokine production as well as expression of activation markers by DCs.

4.1. The innate immune response to UTIs

After infection with UPEC, a rapid TLR4-dependent neutrophil influx occurs (Fig. 2). These neutrophils are critical for early control of bacterial levels in murine infection models [18]. C3H/HeJ mice, which express a mutant TLR4, have increased bacterial titers after infection, with minimal recruitment of neutrophils [19]. The loss of neutrophil recruitment is likely due to the loss of chemokine expression. A similar neutrophil homing defect is observed in UPEC-infected mice deficient in the murine homolog of the IL-8 receptor [20]. While TLR4 has classically been described in terms of expression by bone marrow derived cells (DCs, macrophages, neutrophils), epithelial tumor cell lines from the urinary tract have been shown to express TLR4 and respond to LPS in vitro [21,22]. The importance of epithelial-expressed TLR4 has been shown in vivo using a reciprocal bone marrow transplantation approach [23]. Wild-type mice that have had their bone marrow replaced with marrow from a TLR4 mutant mouse, and thus have TLR4 mutant neutrophils, show defective neutrophil recruitment to the bladder and high bacterial titers 48 h after UPEC infection. This result confirms the importance of TLR4 expression by bone marrow derived cells for effective innate immunity. Interestingly, mice that have TLR4 wild-type bone marrow derived cells but TLR4 mutant epithelial cells show increased bacterial titers 48 h after infection with UPEC and reduced neutrophil influx. These results demonstrate the necessity of the bone marrow derived cells and suggest that the epithelium is augmenting, or even directing, the innate immune response in UTIs.

In addition to immune signaling, the bladder epithelium performs several other functions to assist bacterial clearance. Although extremely inert, the epithelium activates and undergoes a rapid turnover process immediately after bacterial binding [7,15]. The superficial umbrella cells die by an apoptosis-like mechanism and slough off into the bladder lumen. Underlying epithelial cells then activate complex genetic signaling programs, normally repressed, leading to differentiation of the intermediate cells and regeneration of the superficial epithelial layer [7] (Table 1). Rather than the localized turnover event that occurs upon normal superficial sloughing, UPEC infection induces massive exfoliation throughout the epithelium. Exfoliation of the surface cells appears to decrease bacterial titers by removing the bacteria bound to the cells. Further, the bladder epithelial cells, as well as neutrophils, are also known to increase expression of inducible nitric oxide synthase, producing increased levels of

![Fig. 2. The bladder responds to UPEC infection with a massive influx of neutrophils, which facilitate bacterial clearance. A. An immunostained thin section from an infected mouse bladder shows neutrophils homing to the bladder epithelium (top). The neutrophils are stained with GR1 antibody (red), and nuclei are counterstained with Hoescht (blue). Scale bar, 50 µm. B. Once the neutrophils reach the bladder lumen, they swarm over infected cells, as seen in this scanning electron micrograph. Extracellular bacteria are easily eaten, but the IBCs are relatively unharmed. Scale bar, 50 µm.](image)

### Table 1: UPEC-induced changes in bladder genetic regulation *

<table>
<thead>
<tr>
<th>Time (h)</th>
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<th>Expression</th>
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<tr>
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<tr>
<td>168</td>
<td>Wnt5a</td>
<td>Differentiation</td>
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*Activation and repression of factors was determined by qRT-PCR on mRNA isolated from infected mouse bladders at various time points post-inoculation [7].

**Time post-inoculation at which maximal effect was observed.
nitric oxide and its breakdown products in an effort to clear UPEC from the bladder [24].

Recently, a novel TLR (TLR11) was characterized whose expression is restricted to the urinary tract and appears to specifically recognize UPEC [25]. UPEC infection in TLR11-deficient mice results in severely increased bacterial load in the kidneys and dramatically lowered renal inflammation. While the ligand for TLR11 has not been described, the identification of a TLR preferentially expressed in the urinary tract will greatly impact our understanding of the pathogenesis of UTIs.

4.2. Role of adaptive immune responses to UPEC

Relatively little investigation has been performed to identify the role for the adaptive immune response in UTIs. SCID mice, which lack both B and T lymphocytes, appear to be more susceptible to UTIs [26]. However, mice lacking thymus-derived T lymphocytes (nude mice) appear to have a normal response to UTIs [26]. Additionally, mice lacking B lymphocytes due to a chromosomal deletion of a section of the antibody receptor gene complex (JH-deficient mice) also have a normal response to UTIs [27]. Conversely, mice deficient in the γδ-T lymphocyte receptor complex are more susceptible to UTIs [27]. γδ-T lymphocytes are absent in SCID mice, but present in nude mice, and they are thought to be important for epithelial immunity in mucosal tissues. These poorly understood lymphocytes might influence epithelial sloughing and nitric oxide production during the innate response to UTI [27].

5. Subversion of host response

UPEC overcomes this massive immune assault quite effectively. In a mouse model of UTI, UPEC maintains extremely high bacterial titer in the bladder during the first couple of days post-infection [15,28,29]. These levels begin to decline, but the bacteria are not cleared from the bladder. Instead, a significant reservoir of organisms can persist for months in the bladder, even after treatment with antibiotics [30]. Periodically during this persistent phase, high levels of organisms are encountered in the urine. Because UTIs are clinically diagnosed by detecting high bacterial loads in the urine, these spikes in bacteriuria in the mouse may be analogous to recurrences of infection [3]. Recently, it was determined that human subjects with or without an active UTI can secrete viable but nonculturable organisms in their urine [31]. Thus it seems that UPEC is quite adept at creating a protective persistent niche in the urinary tract, which may serve as a seed for recurrent infections.

5.1. Intracellular bacterial communities

It was recently discovered that UPEC undergoes a complex developmental program in the superficial cells of the bladder, whereby it forms intracellular bacterial communities (IBCs) which mature into biofilm-like structures inside individual cells [29,32] (Table 2). This represents the first description of intracellular biofilms in living tissues. Bacterial biofilms generally provide a protective environment for their constituent bacteria, shielding them from antibiotics and host immune effects [33], and it seems quite likely that biofilms in the bladder may account for the recalcitrance of PEC to treatment and clearance from the urinary tract. The IBC program has been dissected using time-lapse videomicroscopy, wherein mouse bladders were infected with GFP-expressing UPEC [32]. Bladders were harvested at various time points, stretched on an incubation chamber, and placed on an inverted epifluorescence microscope, which preserved the viability of the bladder epithelium and allowed visualization of infection in living tissue. Micrographs were taken every 30 s–2 min for several hours at a time. Overlapping time points were chosen, which allowed reconstruction of an infection timeline. This technique revealed that UPEC progresses through several steps, beginning with type 1 pilus-mediated binding of the superficial epithelial cells and invasion into these cells.

Once inside the bladder superficial cells, UPEC divides relatively rapidly in the cytoplasm of the host cell, with a doubling time of approximately 30–35 min [28,32]. These early IBC events result in formation of a small bacterial community inside the bladder cell (Fig. 1B). As they grow, the bacteria maintain their typical rod shape of approximately 3 µm and form a loosely-organized cluster, with microorganisms randomly oriented in the cell cytoplasm. At 6–8 h post-inoculation, several dramatic phenotypic changes take place, whereby the early IBC matures into a biofilm-like...
community [29,32]. During this phenotypic switch, the bacterial growth rate slows to greater than 60 min. Concurrently, bacterial cell length shifts to an average of 0.7 µm, and the entire cluster begins to pack together and form a tight, compact globule, termed a middle IBC [32] (Fig. 1C). Visualization of the surface of infected mouse bladders at these time points reveals the presence of large protrusions, or pods, extending from the bladder surface, each of which represents one superficial epithelial cell filled with thousands of UPEC [29]. The bacteria remain underneath the UP-coated lumenal membrane of the bladder cell, surrounded by the host cell cytoplasm. The bacteria express numerous fibers on their surface, creating a zone around themselves and establishing individualized compartments for each bacterium [29].

The organization and structure of these middle IBCs is highly reminiscent of bacterial biofilms [34]. In fact, in the middle IBC, UPEC expresses several surface molecules also important for E. coli biofilm formation in vitro, namely type 1 pili and antigen 43 [29]. The expression of these molecules is heterogeneous across the community, the bacteria seemingly forming subpopulations within the IBC. Biofilms often establish compartments within the whole structure by differential gene regulation in a process of division of labor and specialization [33]. The bacteria in biofilms also encase themselves in a matrix of polysaccharides, or other polymeric substances [34]. Likewise, the bacteria of the middle IBC are surrounded by polysaccharides as well [29].

Biofilms shield bacteria from harsh environmental challenges, such as antibiotics and the host immune response [33]. Several factors influence this protection, including slower growth rate of the bacteria, physiological changes in the bacteria, expression of factors which inhibit antibiotic activity, and the decreased penetration of the biofilm matrix by antibiotics and immune effectors. In this manner, a biofilm lifestyle results in persistence of bacteria in environmental locations as well as in infected tissues. During UTI, intracellular biofilm-like IBCs also protect UPEC from immune clearance. The hordes of neutrophils that infiltrate the bladder upon infection migrate directly to infected superficial cells, ignoring uninfected cells [32] (Fig. 2). However, the neutrophils are unable to efficiently penetrate the IBC and engulf the bacteria.

Variations in host immune competency may account for different responses to UPEC, and thus different levels of bacterial activity. For instance, earlier studies utilizing powerful microscopic techniques to uncover bacterial interactions in the mouse bladder did not report the presence of intracellular biofilm-like structures [15,28]. These studies, however, used C57BL/6 mice, which exhibit a strong exfoliation response to UPEC infection. Infection of C3H/HeN mice, which have a much milder exfoliation response, leads to numerous pod-like structures representing mature middle IBCs [29]. Interestingly, infection of C3H/HeJ mice results in a significant increase in the number of mature IBCs present in the bladder epithelium, presumably due to an abrogated innate immune response and a lack a neutrophil infiltration [19,29].

Eventually, bacteria on the edge of the IBC detach from the community, differentiate to typical rod morphology, become highly motile, and swim to one point in the host cell [32]. Once they reach the cell edge, the bacteria burst out of the host cell into the bladder lumen in a process termed fluxing [28] (Fig. 1D). Often, these escaped bacteria become highly filamentous, reaching up to 70 µm or greater in length, and these filaments represent the majority of lumenal bacteria by 20 h post-inoculation [32]. However, filaments do not form during the first 48 h of infection in mice with a defect in TLR4, suggesting a connection between filamentation and the immune status of the host [32]. The signals inducing bacterial filamentation remain unknown, but it has been shown that neutrophils have difficulty engulfing and destroying the filaments. Neutrophils can digest rod-shaped bacteria on the bladder surface quite readily, but the filaments remain viable and protected from the assault, even when neutrophils make contact and extend pseudopods around them. In this manner, UPEC survives the immune onslaught associated with infection, thus increasing the chance for re-attachment to the bladder epithelium.

5.2. Persistent bacterial reservoir

Many of the surviving bacteria re-bind the bladder superficial cells, re-invade, and undergo another round of IBC formation, although this subsequent cycle progresses at a slower rate than the first [32]. Multiple successive rounds most likely occur, but after several days post-inoculation, only small intracellular clusters of bacteria survive inside the bladder superficial epithelium. These bacteria appear to be in a dormant state, neither dividing nor progressing through the IBC cascade. It is possible that these quiescent bacteria represent a reservoir of pathogens in the bladder, and activation of these bacteria may lead to a recurrence of UTI.

Many questions remain about IBC formation and evasion of the host response. Most intriguing, perhaps, is that the IBC program has only been seen to progress in the bladder superficial cell, never in the underlying cells [28,29,32]. Recent studies have shown intriguing similarities in signaling behavior between bladder epithelial cells and neuronal cells, including specialized receptors and ion channels that trigger intracellular signals [35]. Signals initiated by bladder epithelial channels may influence bacterial behavior and regulate IBC progression. The underlying cells might also contain an inhibitory signal, which prevents IBC progression. Such an inhibition might account for the switch to a quiescent reservoir state, as more and more fluxing bacteria begin to bind and invade into underlying cells because of superficial cell exfoliation.

6. Concluding remarks

In the clinic, UTI is defined as the presence of bacteria in the urine [3]. By this definition, UTIs are acute ailments:
once the initial symptoms fade, no culturable organisms are present in the urine. However, it appears that UTIs can be more chronic than previously thought, using the bladder as a reservoir for persistence, in addition to the previously characterized vaginal and intestinal reservoirs. The IBC program seeks to establish this reservoir by allowing UPEC to gain a foothold in the urinary tract. In the community, presumably only a relatively few microorganisms gain entry to the bladder, and these would quickly be eliminated by a robust host response if left in the harsh bladder lumen. By invading, UPEC creates a safe haven for itself, a protected, nutrient-rich environment in which to rapidly increase its numbers to high levels [15,28,29,32]. Once high bacterial numbers are achieved, UPEC bursts out into the lumen, and this sudden flood of microorganisms increases the chances of bacterial spread throughout the urinary tract, while allowing significant amounts of bacteria to be transmitted to the environment in search of a new host [28,30]. Considering the complex cascades involved in mounting a successful immune response, and the number of factors influencing immune activity, it is conceivable that slight deficiencies in host pathways may lead to a variety of infection outcomes. In fact, a recent study of inflammatory response in patients with recurrent UTI revealed that neutrophils from these patients displayed reduced levels of CD16, decreased bacterial phagocytosis, and lowered generation of reactive oxygen intermediates [36]. Thus, the myriad of UTI clinical outcomes results from a delicate interplay between the bacterium and host. The bacterial reservoir observed surviving for several months in the bladder might persist because the bacteria appear to achieve dormancy inside the superficial epithelial cells. The host does not respond to these bacteria, and the bacteria are, in turn, not doing anything which requires a response. In this manner, UPEC takes advantage of bladder biology to establish a chronic infection state in the bladder, which, under the right circumstances, may lead to multiple recurrences. Further investigation may reveal IBC-like cascades used by many other pathogens to generate chronic, recurrent infections in other disease states.

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